JOURNAL OF CLINICAL MICROBIOLOGY, July 1995, p. 1797–1803 Vol. 33, No. 7
0095-1137/95/$04.00+0

Differentiation of *Bartonella*-Like Isolates at the Species Level by PCR-Restriction Fragment Length Polymorphism in the Citrate Synthase Gene

A. F. NORMAN,[1] R. REGNERY,[2] P. JAMESON,[3] C. GREENE,[3] AND D. C. KRAUSE[1]*

Department of Microbiology[1] and School of Veterinary Medicine,[3] University of Georgia, Athens, and Centers for Disease Control and Prevention, Atlanta,[2] Georgia

Received 30 January 1995/Returned for modification 21 March 1995/Accepted 18 April 1995

The citrate synthase gene (*gltA*) of *Bartonella henselae* was cloned and sequenced to compare genetic divergence among alpha and gamma branches of the class *Proteobacteria* and to develop enhanced genotypic reagents for *B. henselae* identification. *B. henselae gltA* is 1,293 nucleotides in length and 63 to 66% homologous with corresponding gene sequences of *Rickettsia prowazekii*, *Escherichia coli*, and *Coxiella burnetii*. The observed genetic variability suggests that *gltA* sequences can provide a useful means for studying moderate divergence among related bacteria. Oligonucleotides specific for *B. henselae gltA* were evaluated for the ability to prime PCR amplification within the alpha and gamma branches of the proteobacteria. Under the conditions used, only *B. henselae*, *Bartonella quintana*, and *R. prowazekii* template DNAs yielded amplification products (approximately 380 bp). DNAs from 28 *Bartonella*-like isolates of feline origin were amplified by *B. henselae* primers and analyzed for restriction fragment length polymorphism. The resulting patterns for all 28 isolates were similar or identical to that of the recognized *B. henselae* strain. Current studies are aimed at optimization of PCR conditions for specificity and sensitivity of amplification of *Bartonella* sequences from clinical isolates.

Cat-scratch disease (CSD) is a major cause of unilateral regional lymphadenitis in children and adults (4–6, 11, 16). The Centers for Disease Control and Prevention (CDC; Atlanta, Ga.) estimates that over 24,000 cases of CSD occur annually in the United States, of which approximately 2,000 require hospitalization (11). In most cases, the disease is benign and self-limiting after a period of several weeks to months; hence, many cases of this disease go undiagnosed as those who are infected do not seek medical care (4, 6).

Diagnosis of CSD is hindered by nonspecific clinical manifestations and the lack of simple and reliable diagnostic techniques. For over 30 years, clinical diagnosis of CSD has relied on the following (4): (i) contact with an animal (usually a cat or dog); (ii) lymphadenopathy; (iii) identification of an inoculation site, usually a scratch or primary dermal or eye lesion (17); (iv) positive CSD skin test (15); and (v) multiple abscesses or granulomas in lymph node biopsy specimens. The development of more reliable means for diagnosis has been complicated by confusion about the etiologic agent. Since its first description, a variety of microorganisms, including *Afipia felis* recently (7), have been tentatively linked to CSD. However, more recent findings suggest that *Bartonella henselae* (formerly *Rochalimaea henselae* [2, 3]), an organism closely related to the trench fever agent *Bartonella quintana*, is the agent of CSD (1, 9, 10, 13, 18–20, 22, 24). Furthermore, *B. henselae* is also an agent of undiagnosed fever and bacillary angiomatosis in human immunodeficiency virus-infected and otherwise immunocompromised individuals (13, 19, 26).

Primers corresponding to the citrate synthase gene (*gltA*) of *Rickettsia prowazekii* amplify the corresponding sequence from *B. henselae* by PCR, providing a potentially valuable tool for identification and diagnosis (19, 21). However, this approach would benefit from the use of primers based specifically on the sequence of *B. henselae gltA*. In this study, we cloned and sequenced *B. henselae gltA*. Primers specific for this DNA sequence were evaluated for specificity and sensitivity by PCR-restriction fragment length polymorphism (RFLP) in the detection and identification of *B. henselae*. In addition, this technique was applied to the characterization of putative *B. henselae* isolates from bacteremic cats from a North Carolina barrier island.

* Corresponding author. Phone: (706) 542-2671. Fax: (706) 542-2674. Electronic mail address: DKrause@UGA.CC.UGA.EDU.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ*lac proAB* [F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) was grown at 37°C to log phase in Luria broth containing ampicillin at 50 μg/ml (14). Cultures were stored at −80°C in Luria broth containing 12.5% glycerol. *B. henselae* was grown on heart infusion agar plates with defibrinated rabbit blood in the presence of 5% CO_2 for 7 to 14 days at 35°C. Isolates of *B. henselae* were obtained from bacteremic cats on Ocracoke Grove Island, N.C., and stored at −80°C in brain heart infusion broth.

Enzymes and reagents. Restriction endonucleases, calf intestinal phosphate, *Taq* DNA polymerase, and deoxynucleotide triphosphates were obtained from Promega Corp. (Madison, Wis.). All other chemicals were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Fisher Scientific (Pittsburgh, Pa.).

***B. henselae* DNA extraction.** Two milliliters of TNE (0.5 M Tris, 5 M NaCl, 0.05 M EDTA) was added to each blood agar plate to loosen *Bartonella* colonies, which grow embedded in agar. Colonies were scraped into the suspension, and an aliquot was removed, serially diluted in brain heart infusion broth, and plated (0.1-ml volumes) on blood agar for viable cell counts. Sodium dodecyl sulfate (SDS) and proteinase K were added to the remaining cell suspension at concentrations of 1% and 10 mg/ml, respectively, and incubated overnight at 37°C and then for 2 h at 50°C. Samples were extracted sequentially with 1 volume of phenol saturated with TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), 1 volume of 1:1 phenol and chloroform–isoamyl alcohol (24:1), and 1 volume of chloroform-isoamyl alcohol. DNA was precipitated at −20°C overnight by the addition of 2 volumes of 100% cold ethanol, centrifuged at 2,200 × *g*, washed with 70% ethanol and then 100% ethanol, and incubated at 37°C to dry. Pellets were resuspended in 30 to 50 μl of 10 mM Tris-HCl (pH 8.0) on ice for several hours and stored at 4°C. DNAs from other organisms were kindly provided from the sources listed in Table 1.

Oligonucleotide primers. Oligonucleotide primers used for PCR are shown in Table 2. *R. prowazekii* primers were obtained from the Biotechnology Resource Facility at CDC and from Fisher Scientific. *Rickettsia*-based primers were derived from nucleotide sequences within rickettsial *gltA* (27). The first two characters in each primer designation represent the species from which the nucleotide se-

TABLE 1. Purified DNAs used to test the specificities of oligonucleotide primers under high-stringency annealing conditions

Organism (strain)	Source	Amplification[a]
R. rickettsii (ATCC VR-149)	CDC	−
R. prowazekii (ATCC VR-142)	CDC	+
R. tsutsugamushi (ATCC VR-150)	CDC	−
B. quintana (ATCC VR-358)	CDC	+
B. henselae (ATCC 49882)	CDC	+
B. vinsonii (ATCC VR-152)	CDC	−
B. elizabethae (ATCC 49927)	CDC	−
B. bacilliformis (ATCC 35685)	CDC	−
A. felis (ATCC 53690)	CDC	−
Agrobacterium tumefaciens (Chry5)	S. Pueppke, University of Missouri	−
Mycoplasma pneumoniae (M129)	D. Krause, University of Georgia	−
Borrelia burgdorferi (B31)	F. Gherardini, University of Georgia	−
C. burnetii (Nine Mile, phase I)	L. Mallavia, Washington State University	−
E. coli (SK1592)	A. Summers, University of Georgia	−

[a] With annealing at 51°C.

quence was derived, while characters three and four represent the citrate synthase gene. The numeric label represents either the positive strand (beginning with the position of the first nucleotide) or the negative strand (beginning with the position of the last nucleotide). This system was originally implemented by the Viral and Rickettsial Zoonoses Branch at CDC to provide the species and gene from which each primer was derived and the position and size of each PCR-amplified product (21).

PCR amplification and analysis. PCR amplifications were carried out in 100-μl reaction volumes with the PTC-100 programmable thermal cycler (MJ Research, Inc., Watertown, Mass). A standard PCR reaction mixture consisted of the following: 1 μl of the appropriate DNA template, 1 U of *Taq* DNA polymerase, 10 μl of 10× *Taq* buffer, 6 μl of $MgCl_2$ (25 mM), 0.6 μl of deoxynucleotides (100 mM each), 50 pmol of each primer, and sterile distilled water to a final volume of 100 μl. Ampliwax (Perkin-Elmer Cetus, Branchbur, N.J.) was used to overlay reactions and serve as a vapor barrier. Amplification of *Bartonella* template DNA with *Rickettsia* primers included 35 cycles of denaturation at 95°C for 20 s, annealing of primers at 42°C for 30 s, and primer extension at 60°C for 2 min. Amplification with *Bartonella* primers differed only in annealing and extension temperatures (51 and 72°C, respectively).

Amplified products were examined by agarose gel electrophoresis. Samples were electrophoresed at 110 V for 6 h through horizontal 1% low-melting-point agarose gels, stained with ethidium bromide for 25 min, and viewed with UV light (14).

DNA digestion and electrophoresis. Restriction endonuclease digestions of *B. henselae* chromosomal DNA were incubated at 37°C for 3 h, and the appropriate volume of loading dye (25% of the total sample volume) was added to each sample (14). Restriction profiles were analyzed by using 6-mm-thick, 1% agarose horizontal gels electrophoresed at 110 V for 6 h, stained with ethidium bromide and viewed by UV light (14). In some cases, the appropriate region of interest was excised with a sterile razor blade and purified for cloning procedures with a Gene Clean kit (Bio 101, Inc., La Jolla, Calif.). PCR-RFLP, sensitivity, and specificity samples were analyzed by using Novex vertical Tris-borate-EDTA–4 to 20% acrylamide gels (San Diego, Calif.). Samples were electrophoresed at 100 V for 90 min, and gels were stained with ethidium bromide and viewed by UV light.

Southern blot hybridizations. DNA was transferred to nylon membranes and probed by standard techniques (14). Membranes were incubated at 68°C in prehybridization solution for at least 1 h. The PCR probe generated with *R. prowazekii gltA* primers and *B. henselae* template DNA was excised from agarose gels with a sterile razor blade, purified with a Magic PCR Preps kit (Promega Corp.), and labeled with a Dig DNA labeling and nonradioactive detection kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The labeled probe was denatured by boiling for 10 min, quickly chilled on ice, and then incubated in hybridization solution for at least 6 h at 68°C. Membranes were washed two times for 5 min each with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M Na_3 citrate [pH 7.0])–1% SDS at room temperature and two times for 15 min each with 0.1× SSC–0.1% SDS at 68°C. Immunological detection was carried out according to the manufacturer's protocol (Boehringer Mannheim).

TABLE 2. Oligonucleotide primers specific for *gltA*

Primer	Species	Nucleotide sequence (5′–3′)[a]	Reference(s)
RpCS.877p	*R. prowazekii*	GGGGGCCTGCTCACGGCGG	19, 27
BhCS.781p	*B. henselae*	GGGGaCCaGCTCAtGGtGG	This study
RpCS.1258n	*R. prowazekii*	ATTGCAAAAAGTACAGTGAACA	19, 27
BhCS.1137n	*B. henselae*	AaTGCAAAAAGaACAGTaAACA	This study

[a] Lowercase letters within nucleotide sequences of primers indicate regions of differentiation.

Nucleotide sequencing. Double-stranded DNA sequence data were generated by using the T7 and Sp6 universal primers, as well as custom-synthesized oligonucleotide primers. DNA sequencing was performed with a 373A automated sequencer (Applied Biosystems, Inc., Foster City, Calif.) at the Molecular Genetics Instrumentation Facility of the University of Georgia. Template DNA was purified by the polyethylene glycol precipitation procedure (Applied Biosystems). A dideoxy terminator sequencing kit was used with the Perkin-Elmer Cetus 9600 model thermal cycler. Specific parameters were chosen on the basis of protocols from Applied Biosystems.

Computer-assisted DNA sequence analyses. Intelligenetics and University of Wisconsin Genetics Computer Group (GCG) nucleotide sequence analysis programs were available through the Biological Sequence/Structure Computational Facility of the University of Georgia. The GCG SEQED program was used to enter and edit new sequence data, and double-stranded DNA sequence data were assembled by using the PUBLISH program. Similar DNA sequences were examined for percent similarity by using the GCG GAP program. The Intelligenetics FASTDB program was used to search the GenBank database. Nucleotide alignments of selected citrate synthase genes were generated by using the GCG PILEUP and PRETTY programs.

Nucleotide sequence accession number. The nucleotide sequence of *B. henselae gltA* (see Fig. 2) has been submitted to GenBank (accession number L38987).

RESULTS

Cloning and localization of the *B. henselae gltA* gene. *B. henselae* chromosomal DNA was digested with various restriction endonucleases and analyzed by agarose gel electrophoresis and Southern hybridization with a PCR probe. That probe, generated with *B. henselae* template DNA and oligonucleotide primers specific for *R. prowazekii gltA* (Table 2), identified a single band in each sample (data not shown). Ultimately, a 3.5-kbp *Eco*RV fragment was isolated and cloned into the *Sma*I site of pGem7zf(+) (Promega) to generate plasmid pKV82. Plasmid pKV82 DNA was digested with restriction endonucleases and probed with the PCR product, identifying a 0.5-kbp *Eco*RI-*Hin*dIII fragment (data not shown), as indicated in the restriction map in Fig. 1.

Nucleotide sequence analysis. Following localization of the

FIG. 1. Restriction map of the 3.5-kbp *Eco*RV fragment encoding the *B. henselae gltA* gene. The shaded area and arrow indicate the location and orientation of the *gltA* gene, respectively. E, *Eco*RI; H, *Hin*dIII.

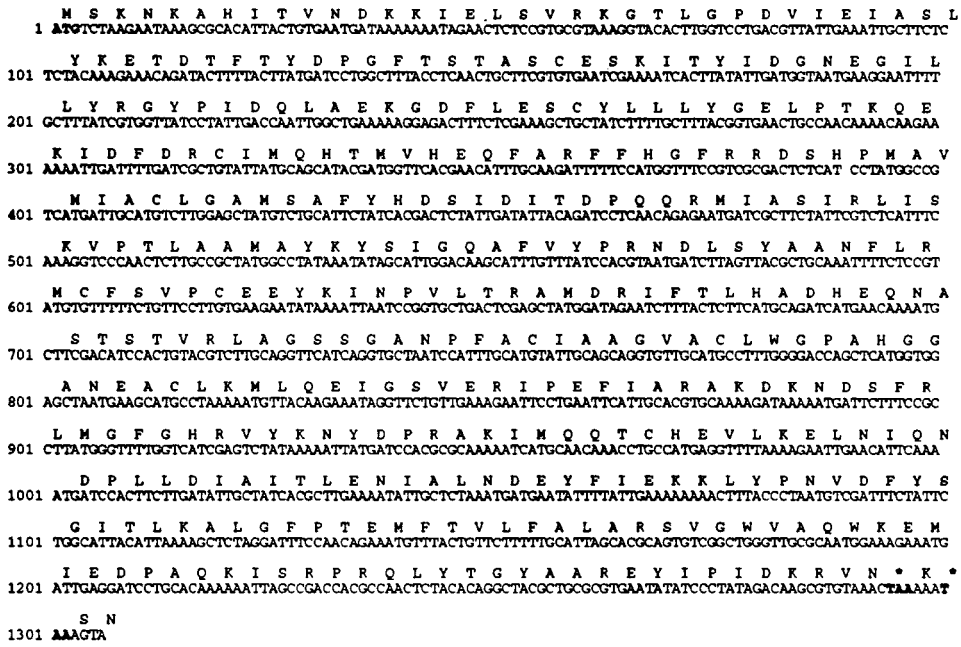

FIG. 2. Nucleotide sequence of the *B. henselae gltA* gene. The sequence of the noncoding strand is shown, with the deduced amino acid sequence indicated above the nucleotide sequence. Asterisks indicate the two TAA stop codons.

B. henselae gltA gene on the 3.5-kbp *Eco*RV fragment, both strands of the complete gene were sequenced (Fig. 2). The *gltA* coding sequence consists of 1,293 nucleotides, beginning with an ATG start codon and terminating with two TAA stop codons. Eight nucleotides upstream of the proposed ATG start codon is an AGG sequence, which may function as the ribosome-binding site for *gltA*. The sequences of BhCS.781p and BhCS.1137n, corresponding to primers specific for *R. prowazekii gltA*, are shown in Table 2. Comparison of *B. henselae* and *R. prowazekii* sequences in the regions corresponding to the forward and reverse primers reveals them to be 78 and 86% identical, respectively (Table 2).

The *gltA* genes of *B. henselae*, *R. prowazekii*, *E. coli*, and *Coxiella burnetii* were aligned to evaluate the extent of nucleotide sequence similarity (Fig. 3). Regions of extensive similarity were observed at the 3′ ends of these genes, and pairwise comparisons with the *B. henselae gltA* gene revealed identities of 65, 66, and 63% with *R. prowazekii*, *E. coli*, and *C. burnetii* sequences, respectively.

Specificity of oligonucleotide primers. The specificity of oligonucleotide primers was evaluated by testing purified DNAs from species of the alpha and gamma proteobacterial groups and randomly selected species as templates (Table 1). The PCR amplification cycle used a stringent annealing temperature (51°C) for specificity. No amplification product was observed for *A. felis*, *Agrobacterium tumefaciens*, *Rickettsia rickettsii*, *Rickettsia tsutsugamushi*, *Bartonella elizabethae*, *Bartonella vinsonii*, *Bartonella bacilliformis*, *Mycoplasma pneumoniae*, or *Borrelia burgdorferi* (data not shown), while a 380- to 400-bp PCR amplification product was observed when *R. prowazekii*, *B. henselae*, or *B. quintana* DNA was used as the template (Table 1 and data not shown).

Genetic variability and sequence divergence of *Bartonella* species were evaluated by comparing the amplification products of *B. henselae*, *B. quintana*, *B. vinsonii*, and *B. elizabethae*. This was accomplished by lowering the primer annealing temperature of the first PCR cycle to 37°C; subsequent cycles were executed at 42°C. Undigested PCR products of *Bartonella* species were approximately 380 to 400 bp (data not shown). PCR-RFLP profiles were generated for all *Bartonella* species as well as a *Bartonella*-like isolate from a bacteremic cat by digesting PCR products with *Taq*I restriction endonuclease. The RFLP profiles of *B. henselae* (Fig. 4, lane 6) and the *Bartonella*-like isolate (Fig. 4, lane 7) were identical, and the restriction fragments migrated with an additive sum of approximately 380 bp.

Sensitivity of PCR-based detection of *B. henselae*. The sensitivity of this PCR analysis was evaluated with 10-fold serial dilutions of *B. henselae* Houston 1 isolate in brain heart infusion broth. An aliquot of each dilution was plated onto blood agar plates and cultured as described above for CFU determinations. The remainder of each dilution was centrifuged at $1,700 \times g$ for 20 min, and cell pellets were resuspended in 50 μl of PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$) and heated for 10 min at 95°C. Twenty-five microliters of each resulting sample was used as a template for PCR under the amplification conditions described in Materials and Methods. By taking into account the volumes used for PCR and viable cell counts, the results indicate a level of detection of 40 organisms from diluted cultures (Fig. 5, lane 8). This assumes that all viable cells were collected by centrifugation and does not take into account the number of nonviable cells in suspensions. PCR amplification products of serial dilution analysis were subsequently digested with *Taq*I, yielding PCR-RFLP profiles equivalent to that of *B. henselae* (data not shown). Finally, analysis of PCR products by Southern hybridization increased the sensitivity of detection by at least 10-fold (data not shown).

RFLP analysis of *Bartonella*-like isolates. A parallel study that entails the characterization of *Bartonella*-like isolates from bacteremic cats by serological methods is in progress. To characterize those isolates further, PCR-RFLP analysis was carried out. Each isolate yielded a PCR product of approximately 380 bp (data not shown). Digestion of PCR products with restriction endonuclease *Taq*I yielded three bands with an additive

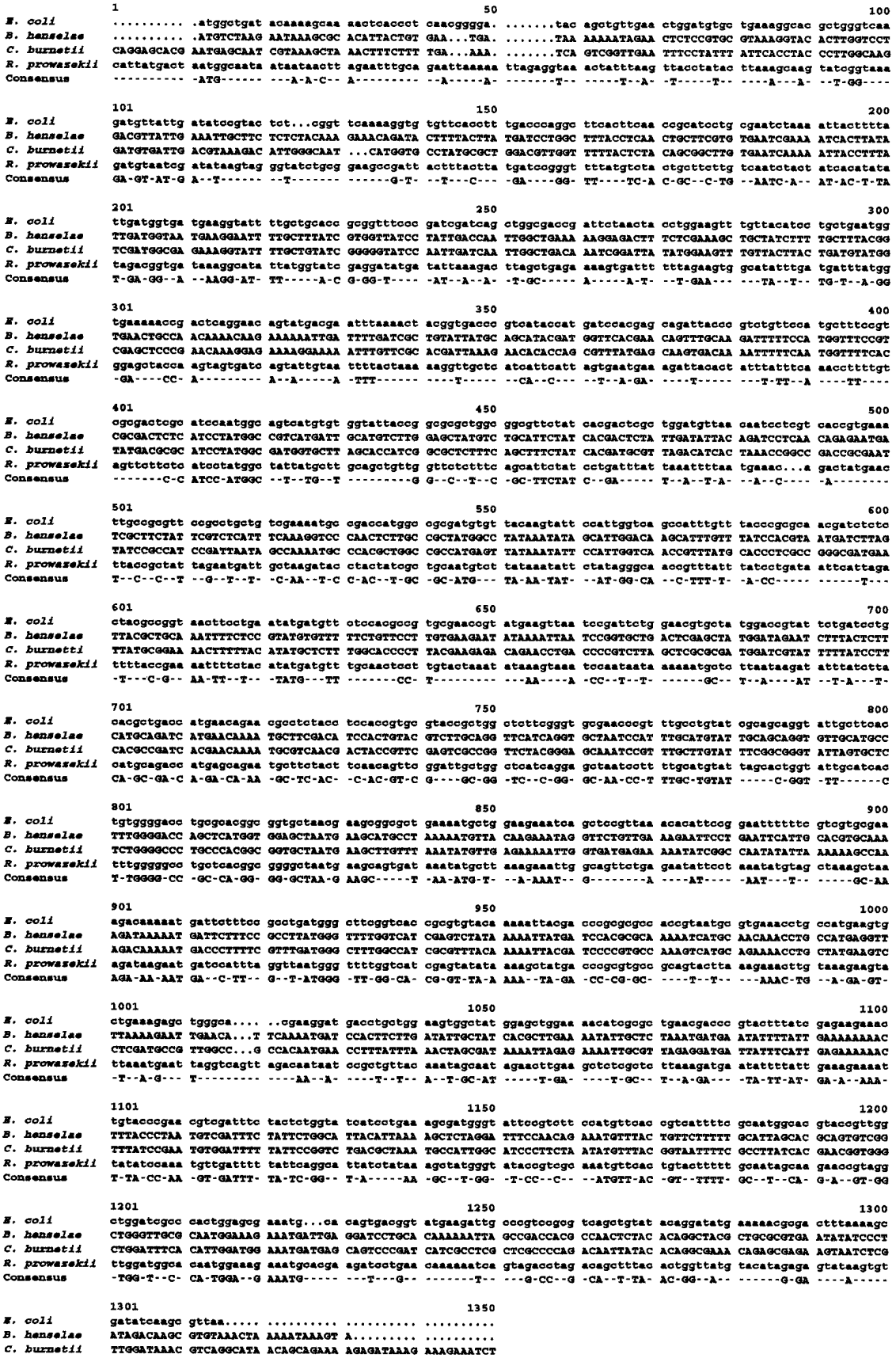

FIG. 3. Nucleotide sequence alignment of selected *gltA* genes. Sequences were aligned and the consensus sequence was computed by using the University of Wisconsin GCG PILEUP and PRETTY programs, respectively. Each dash indicates a lack of consensus among these four sequences. The *B. henselae* sequence is aligned for maximum homology, with dots indicating gaps for alignment purposes.

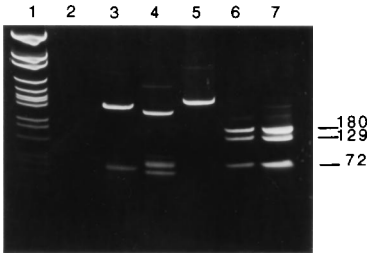

FIG. 4. PCR-RFLP profiles of representative *Bartonella* species. Twenty-microliter aliquots of PCR-amplified products were digested with *Taq*I for 3 h at 65°C and electrophoresed through TBE–4 to 20% acrylamide gels. Gels were stained with ethidium bromide and viewed by UV light. Lanes: 1, pGem molecular weight markers (Promega); 2, negative control (no DNA template); 3, *B. quintana*; 4, *B. vinsonii*; 5, *B. elizabethae*; 6, *B. henselae*; 7, *Bartonella*-like isolate. The sizes of the pGem markers (in base pairs) from top to bottom were 2,645, 1,605, 1,198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, and 36.

sum of approximately 380 bp for each isolate (Fig. 6). The PCR-RFLP profile of each of these isolates was generally identical to that of *B. henselae*. In some isolates, a faint additional band was detected (Fig. 6, lanes 4, 7, 8, and 15). It is not clear whether this reflects some variability within the population or whether it is merely an artifact of PCR analysis. An approximately 40-bp band was also occasionally observed in the lanes of some isolates with both digested and undigested samples (Fig. 6 and data not shown). These bands were also seen with some samples in which no amplification occurred or when template DNA was omitted and probably correspond to a primer-dimer artifact.

DISCUSSION

Until recently, efforts to identify and diagnose CSD have usually relied upon clinical criteria, including (i) contact with an animal (usually a cat); (ii) lymphadenopathy; and (iii) identification of an inoculation site. In some cases, the CSD skin test is also performed and the results are considered in diagnosis. However, the use of CSD skin test antigen made from pasteurized lymph node exudate of another CSD patient is unlicensed for routine use and microbiologically undefined and may pose health risk concerns of its own. To expedite the identification and diagnosis of CSD, bacillary angiomatosis,

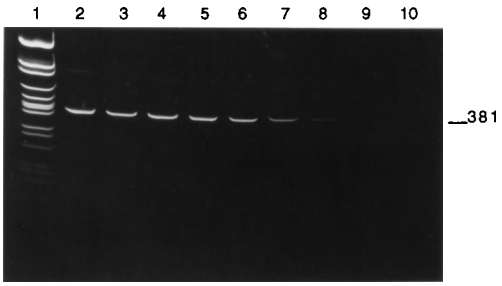

FIG. 5. Serial dilution study to determine PCR sensitivity by using *B. henselae gltA* primers. Lane 1, pGem molecular weight markers (as described in the legend to Fig. 4); lanes 2 through 9, PCR products from 4.0×10^7, 4.0×10^6, 4.0×10^5, 4.0×10^4, 4.0×10^3, 4.0×10^2, 40, and 4 CFU, respectively; lane 10, negative control (no DNA template). Tenfold serial dilutions of *B. henselae* Houston-1 isolate in brain heart infusion broth were made, and aliquots of each dilution were plated onto blood agar plates for viable counts. The remainder of each dilution was prepared for PCR amplification as described in the text. Ten-microliter aliquots of PCR-amplified products were electrophoresed through a TBE–4 to 20% acrylamide gel and then visualized as described in the legend to Fig. 4.

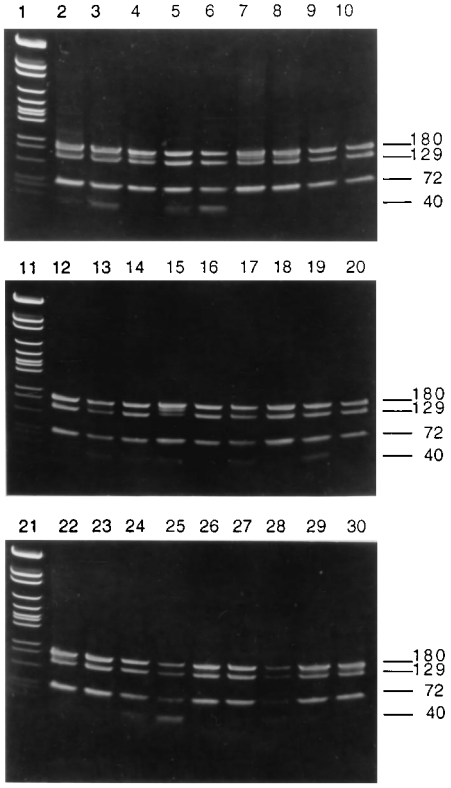

FIG. 6. PCR-RFLP profiles of *B. henselae*-like isolates. PCR-amplified products, obtained with *B. henselae gltA* primers and purified DNA templates of *Bartonella*-like isolates, were digested with restriction endonuclease *Taq*I for 3 h at 65°C. Lanes 1, 11, and, 21, pGem molecular weight standards (as described in the legend to Fig. 4). The remaining lanes correspond to individual isolates. Lanes: 2, J33; 3, J5; 4, J22; 5, F15; 6, J7; 7, F17; 8, NC18; 9, J17; 10, NC53; 12, F5; 13, NCJ21; 14, J13; 15, J28; 16, F25; 17, J24; 18, J20; 19, F10; 20, NCJ30; 22, J31; 23, F23; 24, F37; 25, J6; 26, J12; 27, F36; 28, NC25; 29, F3; 30, NCJ19. Samples (25 μl) were electrophoresed through TBE–4 to 20% acrylamide gels and visualized as described in the legend to Fig. 4.

and related syndromes, methods based on PCR that might allow detection of *B. henselae* in clinical samples have been developed (1, 9, 23). These have included primers corresponding to 16S rRNA sequences (23) and, more recently, the putative *htrA* analog of *B. henselae* (1, 9). In some cases, problems with specificity, including the inability to distinguish *Bartonella* species, have been encountered (1, 8).

Earlier studies by Regnery et al. (19, 21) demonstrated the value of PCR-RFLP by using primers corresponding to *R. prowazekii gltA* to identify and compare divergence among *Rickettsia*-like organisms. One would expect improved sensitivity and specificity from employing oligonucleotides that correspond exactly to *B. henselae gltA* as PCR primers, given that more stringent conditions could be used during primer annealing. Therefore, we sought in this study to sequence the *B. henselae gltA* gene and then to evaluate the products of PCR-RFLP analysis by using various DNA templates, including samples from *Bartonella*-like isolates cultured from bacteremic cats.

The *gltA* coding sequence of *B. henselae* is 1,293 nucleotides in length, encoding a protein of 431 amino acid residues (Fig. 2). The likely N terminus of this protein is predicted on the basis of the location of the ATG relative to a possible ribosome-binding site, as well as comparison of this sequence with *gltA* sequences from other sources. At the protein level, the *B.*

henselae citrate synthase deduced from the nucleotide sequence is similar in size to that of *R. prowazekii* (435 amino acids), *E. coli* (426 amino acids), and pig heart (437 amino acids) (27).

At the nucleotide sequence level, *B. henselae gltA* was similar to the corresponding genes of *R. prowazekii*, *C. burnetii*, and *E. coli* (65, 63, and 66% identity, respectively [Fig. 3]). While nucleotide sequence identities were comparable for all of the bacterial species examined, PCR analysis under stringent conditions with primers specific for *B. henselae gltA* yielded amplification products for only the *B. henselae*, *B. quintana*, and *R. prowazekii* DNA templates (Table 1). Only when the primer annealing temperature was reduced was amplification achieved with *B. vinsonii* and *B. elizabethae* (Fig. 4). Similar specificity was noted in a recent study by Anderson et al. (1) that used primers specific for the *htrA* homolog of *B. henselae* to analyze lymph node tissue from CSD patients by PCR; no amplification products were observed for *B. vinsonii*, *B. bacilliformis*, and *A. felis*, while the amplification product detected for *B. elizabethae* was much larger than expected.

R. prowazekii gltA-based primers have been used in PCR-RFLP analysis of *Bartonella* and rickettsial species (21) and to characterize the type strain (Houston-1) of *B. henselae* (19). As expected, the PCR-RFLP profiles obtained in this study with *B. henselae*-based primers and DNA templates from four *Bartonella* species appeared to be essentially identical to PCR-RFLP profiles obtained previously with *R. prowazekii*-based primers. Furthermore, the PCR-RFLP patterns allowed the differentiation of *Bartonella* species.

Regnery et al. (19) estimated sequence divergence between *B. henselae* and *R. prowazekii* by digesting 381- to 400-bp PCR products of *gltA* with a restriction endonuclease and analyzing comigrating DNA fragments. In that study, only a portion of *gltA* (corresponding to the fragment amplified) was analyzed and an estimated 12.2% divergence was observed between these species. In this study, however, actual sequence divergences of 30% within the amplified products and 35% over the entire *gltA* genes of *B. henselae* and *R. prowazekii* were observed.

Cats have been implicated as a reservoir for *B. henselae* and as a vector in the transmission of CSD and bacillary angiomatosis (10, 11, 16, 18). Regnery et al. (18) isolated *B. henselae* from the blood of a cat that tested positive for *Bartonella*-specific antibody. PCR-RFLP analysis with *R. prowazekii*-based primers yielded patterns identical to that of the Houston-1 type strain of *B. henselae*. In this study, isolates obtained from bacteremic cats were characterized for genetic divergence by PCR-RFLP analysis. All of these isolates had nearly identical profiles (Fig. 6), which supports the identification of them as *B. henselae* isolates. In a parallel study, serologic analysis also supports the identification of them as *B. henselae* isolates. (12). Very minor bands between the 180- and 129-bp *Taq*I products were identified for a few isolates, but it is unclear whether this represents an artifact of the procedure or reflects minor genetic differences in some isolates.

A serologic assay for the diagnosis of *Bartonella*-associated infections has been developed (20). However, this serologic assay (as currently performed) is genus specific, not species specific, when it is applied to human immunoglobulin G. Similarly, this serologic test provides little or no information concerning possible *B. henselae* strain variation.

PCR-RFLP analysis of cultures grown from clinical samples for identification of *B. henselae* is useful for epidemiologic purposes and the research laboratory. Given the time necessary for culture of this slowly growing bacterium, this approach is impractical for rapid identification of isolates in a clinical setting. Direct detection in clinical samples is required to expedite further the diagnosis of CSD. Tzianabos et al. (25) have shown that disease-causing organisms such as *R. rickettsii* can be detected in clinical samples (human tissue and blood) via PCR. In that study, blood samples were obtained from patients diagnosed with Rocky Mountain spotted fever or from healthy patient controls and processed for PCR. Amplification products were subjected to gel electrophoresis and analyzed by staining. The total time to process blood samples to the point of PCR amplification was only 2 to 3 h, while sample preparation, amplification, and visualization of amplified products were accomplished in 1 day. Likewise, Anderson et al. (1) have reported the detection of *B. henselae* from lymph node biopsies of CSD patients by PCR. With improvements in DNA extraction techniques from clinical samples, it should be possible to provide a reliable yet simple means for identifying *B. henselae* infections. The use of defined sequences for PCR-based analyses, as described in this study, offers the additional advantage of ensuring greater reliability and reproducibility, which may not be the case with randomly primed PCR.

In conclusion, this study provides new fundamental information regarding *B. henselae* and its relationship to *Bartonella*-like species. Furthermore, our findings confirm and expand upon previous results on the use of PCR-RFLP analysis for the detection and identification of *B. henselae*. With the primers and technique described, known *Bartonella* species and *Bartonella*-like isolates can be clearly differentiated. Finally, the PCR-RFLP technique has been applied successfully in evaluating isolates from bacteremic cats for *B. henselae*.

ACKNOWLEDGMENTS

We are grateful to P. G. DeBrito, Wake Veterinary Hospital, Inc., for collecting blood samples and to H. Andrianopoulos for technical assistance.

REFERENCES

1. **Anderson, B., K. Sims, R. Regnery, L. Robinson, M. J. Schmidt, S. Goral, C. Hager, and K. Edwards.** 1994. Detection of *Rochalimaea henselae* DNA in specimens from cat scratch disease patients by PCR. J. Clin. Microbiol. **32:**942–948.
2. **Birtles, R. J., T. G. Harrison, N. A. Saunders, and D. H. Molyneux.** 1995. Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. Int. J. Syst. Bacteriol. **45:**1–8.
3. **Brenner, D. J., S. P. O'Connor, H. H. Winkler, and A. G. Steigerwalt.** 1993. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. Int. J. Syst. Bacteriol. **43:**777–786.
4. **Carithers, H. A.** 1985. Cat-scratch disease: an overview based on a study of 1,200 patients. Am. J. Dis. Child. **139:**1124–1133.
5. **Carithers, H. A., C. M. Carithers, and R. O. Edwards, Jr.** 1969. Cat-scratch disease: its natural history. JAMA **207:**312–316.
6. **Dolan, M. J., M. T. Wong, R. L. Regnery, J. H. Jorgensen, M. Garcia, J. Peters, and D. Drehner.** 1993. Syndrome of *Rochalimaea henselae* adenitis suggesting cat scratch disease. Ann. Intern. Med. **118:**331–336.
7. **English, C. K., D. J. Wear, A. M. Margileth, C. R. Lissner, and G. P. Walsh.** 1988. Cat-scratch disease isolation and culture of the bacterial agent. JAMA **259:**1347–1352.
8. **Erler, B. S., A. M. Jiminez, M. L. Gedebou, J. W. Said, and W. S. Nichols.** 1993. Absence of *Rochalimaea henselae* sequences in cat scratch disease lymph nodes using a polymerase chain reaction assay. Mod. Pathol. **6:**105A.
9. **Goral, S., B. Anderson, C. Hager, and K. M. Edwards.** 1994. Detection of *Rochalimaea henselae* DNA by polymerase chain reaction from suppurative nodes of children with cat-scratch disease. Pediatr. Infect. Dis. J. **13:**994–997.
10. **Groves, M. G., and K. S. Harrington.** 1994. *Rochalimaea henselae* infections: newly recognized zoonoses transmitted by domestic cats. J. Am. Vet. Med. Assoc. **204:**267–270.
11. **Jackson, L. A., B. A. Perkins, and J. D. Wenger.** 1993. Cat-scratch disease in the United States: an analysis of three national data bases. Am. J. Public Health **83:**1707–1711.

12. **Jameson, P., and C. Greene.** Unpublished observations.
13. **Koehler, J. E., C. A. Glaser, and J. W. Tappero.** 1994. *Rochalimaea henselae* infection: a new zoonosis with the domestic cat as reservoir. JAMA **271:**531–535.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Margileth, A. M.** 1992. Cat scratch disease and nontuberculous mycobacterial disease: diagnostic usefulness of PPD-battery, PPD-T and cat scratch skin test antigens. Ann. Allergy **68:**149–154.
16. **Margileth, A. M., and G. F. Hayden.** 1993. Cat scratch disease from feline affection to human infection. N. Engl. J. Med. **329:**53–54.
17. **Margileth, A. W., D. J. Wear, T. L. Hadfield, C. J. Schlagel, G. T. Spigel, and J. E. Muhlbauer.** 1984. Cat-scratch disease bacteria in skin at the primary inoculation site. JAMA **252:**928–931.
18. **Regnery, R., M. Martin, and J. Olson.** 1992. Natural occurring *Rochalimaea henselae* infection in domestic cat. Lancet **340:**557–558. (Letter.)
19. **Regnery, R. L., B. E. Anderson, J. E. Clarridge III, M. C. Rodriguez-Barradas, D. C. Jones, and J. H. Carr.** 1992. Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. J. Clin. Microbiol. **30:**265–274.
20. **Regnery, R. L., B. A. Perkins, J. G. Olson, and W. Bibb.** 1992. Serological response to "*Rochalimaea henselae*" antigen in suspected cat-scratch disease. Lancet **339:**1443–1445.
21. **Regnery, R. L., C. L. Spruill, and B. D. Plikaytis.** 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J. Bacteriol. **173:**1576–1589.
22. **Relman, D. A., P. W. Lepp, K. N. Sadler, and T. M. Schmidt.** 1992. Phylogenetic relationships among the agent of bacillary angiomatosis, *Bartonella bacilliformis*, and other alpha-proteobacteria. Mol. Microbiol. **6:**1801–1807.
23. **Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins.** 1990. The agent of bacillary angiomatosis. N. Engl. J. Med. **323:**1573–1580.
24. **Tompkins, D. C., and R. T. Steigbigel.** 1993. *Rochalimaea*'s role in cat scratch disease and bacillary angiomatosis. Ann. Intern. Med. **118:**388–390.
25. **Tzianabos, T., B. E. Anderson, and J. E. McDade.** 1989. Detection of *Rickettsia rickettsii* DNA in clinical specimens by using polymerase chain reaction technology. J. Clin. Microbiol. **27:**2866–2868.
26. **Welch, D. F., D. A. Pickett, L. N. Slater, A. G. Steigerwalt, and D. J. Brenner.** 1992. *Rochalimaea henselae* sp. nov., a cause of septicemia, bacillary angiomatosis, and parenchymal bacillary peliosis. J. Clin. Microbiol. **30:**275–280.
27. **Wood, D. O., L. R. Williamson, H. H. Winkler, and D. C. Krause.** 1987. Nucleotide sequence of the *Rickettsia prowazekii* citrate synthase gene. J. Bacteriol. **169:**3564–3572.